Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated on Ser[157] by protein kinase C-dependent and -independent mechanisms in thrombin-stimulated human platelets

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VASP (vasodilator-stimulated phosphoprotein) is an actin- and profilin-binding protein that is expressed in platelets at high levels and plays a major role in negatively regulating secretory and adhesive events in these cells. VASP is a major substrate for cAMP- and cGMP-regulated protein kinases and it has been shown to be directly phosphorylated on Ser[157] by PKC (protein kinase C). In the present paper, we show that, in human platelets, VASP is phosphorylated by PKC on Ser[157], but not Ser[239], in response to phorbol ester stimulation, in a manner blocked by the PKC inhibitor BIM I (bisindolylmaleimide I). In response to thrombin, VASP was also phosphorylated on Ser[157], but this response was only partially inhibited by BIM I, indicating PKC-dependent and -independent pathways to VASP phosphorylation by thrombin. Using inhibitors, we have ruled out the possibility that the PKC-independent pathway acts through guanylate cyclase generation of cGMP, or through a phosphoinositide 3-kinase-dependent kinase. Inhibition of Rho kinase, however, substantially reduced Ser[157] VASP phosphorylation, and its effects were additive with BIM I. This implicates Rho kinase and PKC as the major kinases that phosphorylate VASP Ser[157] in response to thrombin in platelets.

Key words: bisindolylmaleimide I (BIM I), platelet, protein kinase C (PKC), Rho kinase, thrombin, vasodilator-stimulated phosphoprotein (VASP).

INTRODUCTION

The multifunctional organizer of the actin cytoskeleton VASP (vasodilator-stimulated phosphoprotein), together with *Drosophila* Ena (Enabled), is the original member of the Ena/VASP family, which consists further of *Caenorhabditis elegans* Unc-34, *Dictyostelium* DbVASP and the other mammalian members Mena (mammalian Ena) and EVL (Ena/VASP-like) protein. VASP was originally isolated from platelets, and, in response to vasodilating agents, such as PGI_2 (prostaglandin I_2) and NO (nitric oxide), which elevate cAMP and cGMP respectively, it was reported that a protein of approx. 50 kDa became phosphorylated [1]. The 50-kDa protein was then purified and characterized as VASP [2], which has been shown subsequently to be widely expressed in other cell types, including neuronal cells, T cells, macrophages, endothelial cells, smooth muscle cells and fibroblasts [3,4]. Platelets are reported, however, to express approx. 78 000 copies/platelet [5], levels which are higher than in most other cells. Halbrugge and Walter [6] have previously quantified the amount of VASP in platelets as 2.45 μg/mg, 5–8-fold more VASP per mg than in human dermal fibroblasts [7], for example.

Ena/VASP family proteins have a highly conserved structure, consisting of an N-terminal EVH1 (Ena/VASP-homology-1) domain, a central proline-rich region and a C-terminal EVH2 domain. The proline-rich region of Ena/VASP proteins is the least conserved domain and allows the physical interaction of VASP with profilin, a 14 kDa G-actin-binding protein, and proteins with SH3 (Src homology 3) and WW domains (protein–protein interaction domains containing two conserved tryptophan residues), including the SH3 domains of Lyn, Src and Fyn *in vitro* [8–10]. VASP itself has been shown to bind the Abl SH3 domain *in vitro* and *in vivo* [11], although a precise role for VASP interaction with SH3-domain-containing proteins remains unclear. However, it is possible that SH3 domains may recruit Ena/VASP proteins to subcellular locations during actin modulation, which may facilitate signal transduction.

In vivo studies by several groups have indicated that VASP negatively regulates platelets. Hauser et al. [12] have shown that in VASP$^{-/-}$ mice, although there was hyperplasia of megakaryocytes in bone marrow and spleen, the blood platelet count in VASP$^{-/-}$ mice was equivalent to that in wild-type mice. In response to thrombin, a greater level of activation was observed in VASP$^{-/-}$ platelets than in wild-type, where VASP$^{-/-}$ platelets exhibited greatly enhanced surface P-selectin expression and fibrinogen binding to integrin αIIbβ3. Aszodi et al. [13] have reported that VASP$^{-/-}$ platelets show significantly enhanced binding to fibrinogen after activation with collagen. Massberg et al. [14] demonstrated that VASP$^{-/-}$ platelets showed increased adhesion to endothelial cells from carotid artery endothelium and denuded endothelium. Furthermore, they showed that VASP$^{-/-}$ platelets were unresponsive to NO$^{\bullet}$, highlighting an essential role for VASP in this inhibitory pathway. The function of VASP in the dynamic rearrangement of the actin cytoskeleton is controversial and remains unclear. Ena/VASP proteins play a critical role in cell motility, migration and adhesion, and VASP may function to promote profilin recruitment, actin nucleation, bundling and filament formation and may also play an anti-branching and anti-capping role [8,15]. VASP has been shown to localize to the leading edge of lamellipodia, actin stress fibres, filopodial tips and to focal adhesions, such as those involving integrin αIIbβ3 in platelets [16,17]. It is postulated that VASP localization in the cell

Abbreviations used: BIM I, bisindolylmaleimide I; Ena, Enabled; eNOS, endothelial NO (nitric oxide) synthase; EVH1, Ena/VASP-homology-1; EVL, Ena/VASP-like; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 8-pCIPhs-cGMP, 8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate; PG, prostaglandin; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; sGC, soluble guanylate cyclase; SH3, Src homology 3; TBSt, Tris-buffered saline with Tween; VASP, vasodilator-stimulated phosphoprotein.

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may be dependent on the binding of VASP to zyxin and vinculin, via its EVH1 domain [18,19].

VASP is a major substrate of PKA (protein kinase A) and PKG (protein kinase G), which phosphorylate it at three sites: Ser^{157}, Ser^{239} and Thr^{278}. These sites are phosphorylated, with differing kinetics, both *in vitro* and in intact human platelets [20]. PKA shows similar *in vitro* and *in vivo* kinetics, with Ser^{157} being the preferred site of phosphorylation, followed by Ser^{239}. PKG, on the other hand, shows differing *in vitro* and *in vivo* kinetics, since, *in vitro*, Ser^{239} is the preferred phosphorylation site, whereas *in vivo*, PKG phosphorylates both Ser^{157} and Ser^{239} with similar kinetics. Thr^{278} is phosphorylated by both PKA and PKG, but only after Ser^{157} and Ser^{239} have been phosphorylated. Phosphorylation of VASP on Ser^{157}, but not on Ser^{239} or Thr^{278}, results in an apparent mobility shift from 46 to 50 kDa on SDS/10 % PAGE, and therefore a mobility shift is seen as a marker of Ser^{157} VASP phosphorylation [21]. The function of the phosphorylation sites on VASP are still not clearly defined, however. Phosphorylation of Ser^{157}, in response to vasodilating agents, correlates with reduced activation of integrin αIIbβ3 and an inhibition of platelet aggregation [22]. In addition, phosphorylation of VASP reduces its ability to interact with actin, thereby negatively regulating its actin nucleation and bundling properties [23].

Recently, it has been shown that PKA and PKG are not the only kinases able to phosphorylate VASP, but that PKC (protein kinase C) may also have this ability [24]. Chitaley et al. [24] reported that, in cultured rat aortic smooth muscle cells, VASP becomes phosphorylated at Ser^{157}, but not at Ser^{239}, in a PKG- and PKA-independent manner in response to the phorbol ester PMA or in response to serum. They conclude that VASP is a substrate of PKC and provide evidence for the involvement of a classical PKC isoform in this phosphorylation [24]. Given the critical roles played by both VASP and PKC in human platelets in regulating key cellular activities, it was important to address whether this novel regulation of VASP by PKC occurred in these cells. In the present study, we set out to determine whether VASP is phosphorylated in platelets upon stimulation with thrombin and to determine the involvement of PKC or other kinase-dependent pathways in this process.

EXPERIMENTAL

Materials

Rabbit polyclonal anti-VASP antiserum was from Alexis Biochemicals (Nottingham, Notts., U.K.). Mouse monoclonal anti-pSer^{239}-VASP antibody (clone 16C2) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-pSer^{157}-VASP antibody was from Cell Signaling Technology (Beverly, MA, U.S.A.). FITC-conjugated anti-(rabbit IgG) was from Molecular Probes (Eugene, OR, U.S.A.). The following were from Calbiochem (La Jolla, CA, U.S.A.): the NO donor diethylamine NONOate; the membrane-permeant cGMP analogue triethylammonium salt, 8-pClPhs-cGMP [8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate]; the sGC (soluble guanylate cyclase) inhibitor, ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one); and the Rho kinase inhibitors Y-27632 and H-1152P. PMA, Gö6976, BIM I (bisindolylmaleimide I) and wortmannin were obtained from Tocris (Avonmouth, Bristol, U.K.) and ECL® (enhanced chemiluminescence) kits were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other reagents were of analytical grade.

Preparation of human platelets

Human blood was drawn from healthy drug-free volunteers on the day of the experiment. ACD (acid citrate dextrose: 120 mM sodium citrate, 110 mM glucose and 80 mM citric acid, used at 1:7, v/v) was used as anticoagulant. Platelet-rich plasma was prepared by centrifugation at 200 ***g*** for 20 min, and platelets were then isolated by centrifugation for 10 min at 400 ***g***, in the presence of PGE_1 (40 ng/ml). The pellet was resuspended to a density of 4×10^8 platelets/ml in a modified Tyrode's Hepes buffer (145 mM NaCl, 2.9 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$ and 5 mM glucose, pH 7.3). To this platelet suspension, 10 μM indomethacin was added, and a 30 min rest period was allowed before stimulation.

Electrophoresis of proteins and immunoblotting

SDS/PAGE and immunoblotting were carried out as described previously [25]. In summary, proteins were separated by discontinuous SDS/PAGE on 10 % slab gels and transferred on to a PVDF membrane by semi-dry transfer (15 V for 1 h). Membranes were blocked by incubation with either 5 % (w/v) BSA (phospho-specific antibodies) in TBSt (Tris-buffered saline with Tween: 150 mM NaCl, 10 mM Trizma and 0.1 % (v/v) Tween 20, pH 7.6) or 5 % (w/v) non-fat dried milk powder in TBSt. Primary antibodies were diluted in either 5 % (w/v) BSA (phospho-specific antibodies) in TBSt, or 2.5 % (w/v) non-fat dried milk powder in TBSt. After incubation with primary antibodies, membranes were washed with TBSt and incubated with horseradish-peroxidase-conjugated secondary antibodies, diluted in 3 % (w/v) BSA in TBSt, for 1 h at room temperature (20 °C). After further washing of the membrane, signals were detected by ECL®. When necessary, membranes were stripped using Restore Western blot stripping buffer (Pierce, Perbio Science, Cheshire, U.K.). Membranes were immersed in stripping buffer and incubated at room temperature for 30 min, before extensive washing and re-probing with the appropriate antibody. Phosphorylation of VASP at Ser^{239} induces no change in apparent molecular mass [24] and therefore this event was assessed only by immunoblotting with phosphospecific anti-pSer^{239} antibody. Phosphorylation of VASP at Ser^{157}, however, induces an increase in apparent molecular mass [24], and therefore may be assessed either by immunoblotting with phosphospecific anti-pSer^{157} antibody or by assessment of the shift in molecular mass by immunoblotting with anti-VASP antibody. These two approaches are therefore used for assessment of Ser^{157} phosphorylation in the present study.

Immunofluorescence confocal imaging

Platelets were prepared as described above, before being pretreated with antagonists and stimulated. Reactions were terminated by addition of 4 % (w/v) paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.0). Platelets were pelleted by centrifugation at 2500 ***g*** for 2 min in a microcentrifuge and washed twice in PBS. Platelets were immobilized on poly(L-lysine)-coated coverslips overnight, permeabilized by incubation of coverslips with 0.05 % (v/v) Triton X-100/PBS at room temperature for 10 min, and incubated for 30 min at room temperature with 1 % (w/v) BSA in PBS, to block non-specific antibody binding. Samples were then incubated for 3 h in 1 % (w/v) BSA/PBS at room temperature with primary antibodies, coverslips washed in 0.05 % (w/v) Triton X-100/PBS and incubated for 30 min at room temperature with 1 % (w/v) BSA in PBS. FITC-labelled anti-(rabbit IgG) secondary antibody was then added at a concentration of 4 μg/ml in 1 % (w/v) BSA in PBS, for 1 h at room temperature. Subsequently, coverslips were washed four times in 0.05 % (w/v) Triton X-100/PBS and mounted on to slides using a 13.5 % Mowiol solution containing 2.5 % DABCO (1,4-diazadicyclo[2.2.2]octane) to prevent bleaching of fluorescence. Platelets were imaged using

a Leica TCS-NT confocal laser-scanning microscope equipped with a Kr/Ar laser attached to a Leica DM IRBE inverted epifluorescence microscope with phase-contrast.

Quantification of data and statistical analysis

Western blots were analysed by densitometry using a Kodak EDAS290 gel documentation system, and density of bands was measured using Scion Image software. Data are normalized to the maximal phosphorylation of VASP within each experiment, and displayed as means ± S.E.M. Significance of difference was analysed by ANOVA with Bonferroni post-test.

RESULTS

VASP is phosphorylated on Ser^{157} in a PKC-dependent manner

Chitaley et al. [24] have demonstrated recently that acute treatment of rat cultured aortic smooth muscle cells with 1 μM PMA for 30 min resulted in the phosphorylation of VASP on Ser^{157}, as detected with a polyclonal anti-VASP antibody. This was an important finding, since it implied that PKC might regulate VASP function directly and thereby regulate the actin cytoskeleton through VASP. It was important to show that this effect could also be seen in a primary human cell; Figure 1 shows the effect of PMA on VASP phosphorylation in human platelets. Stimulation with 1, 3 or 10 nM PMA did not induce phosphorylation of VASP, but higher concentrations of 30, 100, 300 and 1000 nM did induce phosphorylation, as assessed by a shift in the apparent molecular mass, seen when immunoblotted with polyclonal anti-VASP antibody (Figure 1A). This shift in apparent molecular mass is associated with phosphorylation of Ser^{157}, as confirmed by Figure 1(B), where a specific anti-pSer^{157}-VASP antibody was used. Figure 2(A, i) shows that stimulation of platelets with 100 nM PMA led to a time-dependent phosphorylation of VASP, where phosphorylation was not evident at 1 min, but a marked VASP phosphorylation was detectable 5 and 15 min post-stimulation. Importantly, this was paralleled using an anti-pSer^{157} antibody (Figure 2A, ii), but no detectable phosphorylation at Ser^{239} (Figure 2A, iii) could be seen. The PKC inhibitor, BIM I, completely abolished Ser^{157} phosphorylation, as shown in Figure 2(A, ii). Interestingly, Chitaley et al. [24] used the classical isoform-selective inhibitor of PKC, Gö6976, to demonstrate that the major PKC isoforms involved in the response are classical isoforms. We were also able to show that, at 1 μM, Gö6976 blocked phosphorylation of VASP in human platelets in response to PMA (100 nM) (results not shown), suggesting primarily a role for classical isoforms in this response. By way of positive controls, treatment of platelets with PGE_1 (which induces a rise in cAMP) or the cGMP analogue, 8-pClPhs-cGMP, which activate PKA and PKG respectively, induced phosphorylation of both Ser^{157} and Ser^{239} (Figures 2A, ii, and 2A, iii), although, at low concentrations, 8-pClPhs-cGMP selectively induced phosphorylation of Ser^{239}. Importantly, we also showed that the PKC inhibitor BIM I had no effect upon phosphorylation of VASP induced by 8-pClPhs-cGMP (Figure 2B).

Thrombin induces phosphorylation of VASP at Ser^{157} by PKC-dependent and PKC-independent mechanisms

Having demonstrated that VASP could be phosphorylated in platelets at Ser^{157}, but not at Ser^{239}, in a PKC-dependent manner, we were interested in determining whether a physiological agonist that couples to activation of PKC could also induce VASP phosphorylation. Figure 3(A) shows that stimulation of platelets with 0.1 units/ml thrombin results in phosphorylation of VASP to a

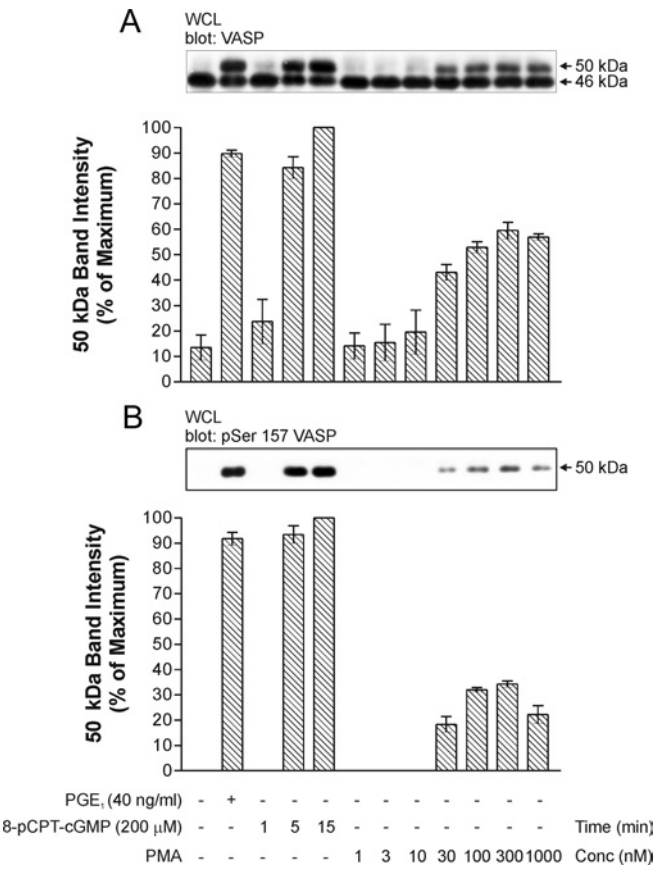

Figure 1 Phorbol ester induces phosphorylation of platelet VASP on Ser^{157}

Human platelets were stimulated with various concentrations of PMA (as indicated) for 5 min in the presence of 1 mM EGTA. Reactions were stopped by the addition of 5-fold sample buffer, and proteins were separated by SDS/PAGE. Samples were immunoblotted with polyclonal anti-VASP antibody (**A**) and anti-pSer^{157} VASP-antibody (**B**). As a positive control for VASP phosphorylation, platelets were pre-incubated with either PGE_1 (40 ng/ml) for 3 min or 8-pClPhs-cGMP (8-pCPT-cGMP; 200 μM) for 1, 5 or 15 min, as indicated. Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry and represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pClPhs-cGMP (200 μM) for 15 min. Results are means ± S.E.M. ($n = 3$). WCL, whole-cell lysate.

degree similar to that shown in response to PMA. Importantly, however, although the response to PMA is abolished in the presence of the PKC inhibitor BIM I, the response to thrombin is only partially diminished. The partial nature of the inhibition by BIM I in response to thrombin is not due to the presence of insufficient inhibitor, since an inhibition–response relationship shows maximal effectiveness with 20 μM BIM I (Figure 3B). All experiments described in the present study were conducted in the presence of EGTA (1 mM) in order to block activation of integrin αIIbβ3. Figure 3(C) shows that thrombin-induced VASP phosphorylation in the presence of EGTA is identical with its phosphorylation in the presence of the selective αIIbβ3 integrin antagonist RGDS (Arg-Gly-Asp-Ser) peptide (100 μg/ml). Both conditions abolished platelet aggregation induced by thrombin (results not shown), and, from this, we infer that the phosphorylation of VASP observed in the present study is not secondary to activation of the integrin. As shown for PMA, thrombin induced phosphorylation of VASP at Ser^{157}, but not at Ser^{239}. This is seen by immunoblotting with polyclonal anti-VASP antibody (Figure 4i), anti-pSer^{157}-VASP antibody (Figure 4ii) and anti-pSer^{239}-VASP antibody (Figure 4iii). PMA-induced VASP phosphorylation on Ser^{157} was again completely abolished by BIM I, whereas thrombin-induced

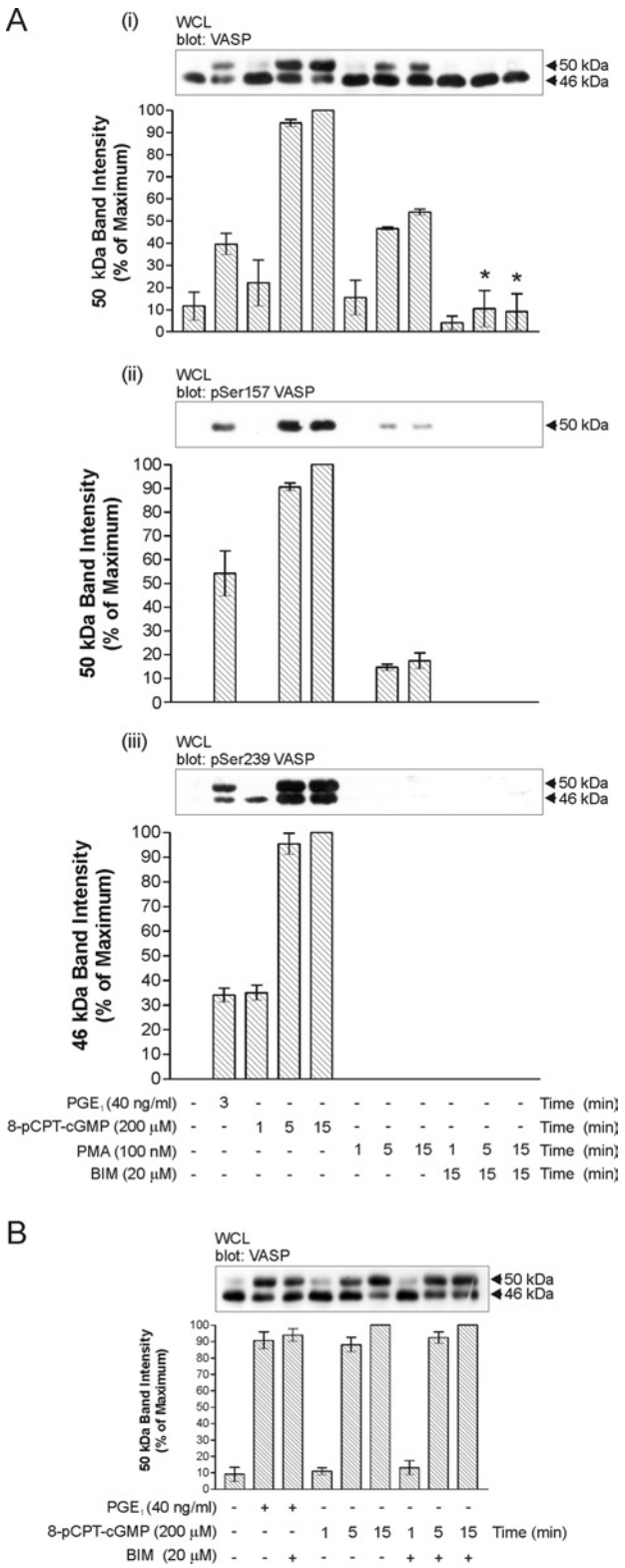

Figure 2 Phosphorylation of VASP by PMA is PKC-dependent

Platelets were pre-treated with BIM I (20 μM) or DMSO as vehicle control for 15 min before stimulation with PMA (100 nM) for 1, 5 or 15 min in the presence of 1 mM EGTA. As positive controls for VASP phosphorylation, platelets were pre-incubated with PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 1, 5 and 15 min, as indicated. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were separated by SDS/PAGE. VASP phosphorylation was detected by immunoblotting with polyclonal anti-VASP antibody (**A**, i and **B**), anti-pSer^{157}-VASP antibody (**A**, ii) and anti-pSer^{239}-VASP antibody (**A**, iii). *, $P < 0.05$ compared with the respective PMA alone controls. (**B**) Platelets were pre-treated with BIM I (20 μM) or DMSO as vehicle control for 15 min before treatment with either PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (200 μM) for 1, 5 or 15 min. VASP was detected by immunoblotting with polyclonal anti-VASP antibody. Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 15 min. Results are means ± S.E.M. ($n = 3$). WCL, whole-cell lysate.

VASP phosphorylation on Ser^{157} was only partially inhibited by BIM I, suggesting the presence of PKC-dependent and PKC-independent mechanisms of VASP phosphorylation by thrombin. These results were paralleled by results observed when using the classical PKC isoform inhibitor Gö6976, which also resulted in a partial attenuation of Ser^{157} VASP phosphorylation (results not shown).

Thrombin-induced phosphorylation of VASP does not involve PKG or PI3K (phosphoinositide 3-kinase), but does involve activation of Rho kinase

It was important to determine the mechanism underlying PKC-independent phosphorylation of VASP Ser^{157} by thrombin. Platelets express an eNOS (endothelial NO synthase), which is able to generate NO in response to some platelet agonists, as has been reported previously for ADP, collagen and thrombin [25a,25b]. Activation of platelets with these agonists can lead to a rise in platelet cGMP levels [26] through Ca^{2+}-dependent calmodulin activation of eNOS. To rule out the possibility that thrombin-induced VASP phosphorylation was being mediated by an NO$^{\bullet}$/cGMP/PKG pathway, we used the well-characterized inhibitor of guanylate cyclase, ODQ [27]. For control purposes, ODQ (10 μM) was able to block the ability of the NO$^{\bullet}$ donor, NONOate (10 μM), to induce phosphorylation of VASP, both at Ser^{157} and at Ser^{239} (Figure 5A). In contrast, Figure 5(B, i) shows that, when platelets were pre-incubated with ODQ, there was no inhibition in the level of thrombin-induced VASP phosphorylation on Ser^{157}, as detected by the shift in the apparent molecular mass using polyclonal anti-VASP antibody. In agreement with data shown in Figure 4(iii), thrombin did not cause any detectable phosphorylation of VASP on Ser^{239} (Figure 5B, ii).

Activation of PKG could not therefore account for the PKC-independent phosphorylation of VASP Ser^{157} by thrombin. It was possible, however, that, since thrombin induces activation of PI3K [28,29], with subsequent activation of multiple protein kinases, including PKB (protein kinase B)/Akt, that this pathway was partly responsible for phosphorylation of VASP. When platelets were pre-incubated with wortmannin (100 nM) for 15 min, there was no reduction in thrombin-induced VASP phosphorylation (Figure 6), indicating a lack of involvement of PI3K-dependent pathways in this event. It has been demonstrated previously, however, that thrombin activates p160 Rho kinase in platelets [29,30]. Figure 7 shows the effect of two structurally distinct Rho kinase inhibitors, H1152P and Y27632, on thrombin-induced phosphorylation of VASP Ser^{157}. Figure 7(A) shows the effect of increasing concentrations of Y27632 on the phosphorylation of VASP, indicating that at concentrations of 10 μM, used in subsequent experiments, the inhibitor is maximally effective. In the presence of either Y27632 (10 μM) (Figure 7B) or H1152P (10 μM) (Figure 7C), phosphorylation of VASP Ser^{157} was partially ablated to a level comparable with that of inhibition by BIM I. Moreover, when platelets were pre-incubated with either of the Rho kinase inhibitors in combination with BIM I,

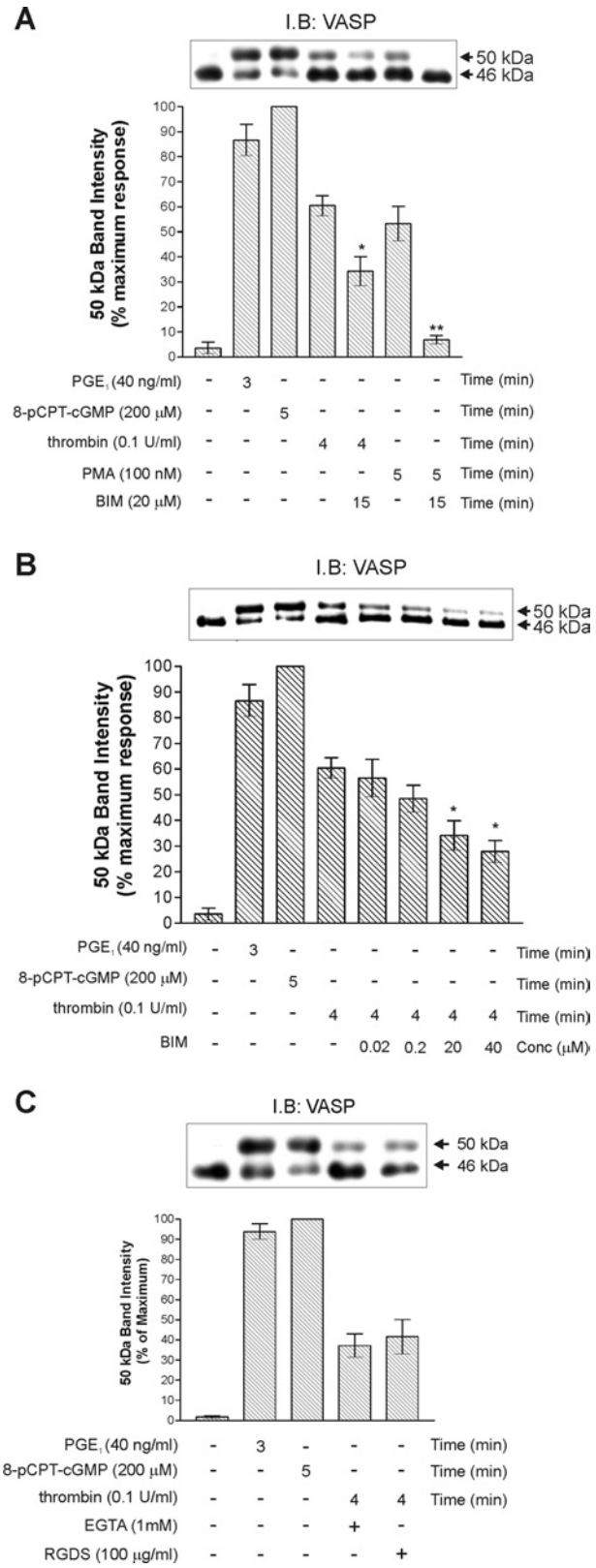

Figure 3 Thrombin induces phosphorylation of VASP in a manner partially inhibited by BIM I and independent of platelet aggregation

(**A**, **B**) Platelets were pre-treated with BIM I (20 μM) or DMSO as vehicle control for 15 min before stimulation with PMA (100 nM) for 5 min or thrombin (0.1 units/ml) for 4 min in the presence of 1 mM EGTA. (**C**) Platelets were pre-incubated either with EGTA (1 mM) for 60 s or with RGDS peptide (100 μg/ml) for 5 min before stimulation with thrombin (0.1 units/ml) for 4 min. As positive controls for VASP phosphorylation, platelets were pre-incubated with PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 5 min, as indicated. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were separated by SDS/PAGE. VASP phosphorylation was detected by immunoblotting (I.B) with polyclonal anti-VASP antibody. Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 5 min. Results are means $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ compared with thrombin alone control; **, $P < 0.05$ compared with PMA alone control.

thrombin-induced VASP phosphorylation on Ser^{157} was more substantially diminished, although the small degree of residual phosphorylation may indicate a role for a third unidentified kinase. These data suggest a role for Rho kinase in mediating PKC-independent phosphorylation of VASP Ser^{157} by thrombin.

VASP translocates in a PKC- and Rho kinase-dependent manner upon stimulation of platelets

Although it was not possible to address definitively the role of phosphorylation of Ser^{157} in these experiments, it was possible to address the role played by PKC and Rho kinase in mediating VASP translocation. In platelets, VASP has been shown to associate with microfilaments and focal contacts, and, when allowed to spread on glass, VASP concentrates with actin-rich structures of the terminal portion of radial microfilament bundles [31]. Using confocal immunofluorescence imaging (Figure 8), we found that VASP had a diffuse cytoplasmic staining pattern in basal platelets, and, upon stimulation with PMA (100 nM) for 5 min, VASP translocated to a more peripheral location that was more punctate in appearance, consistent with movement to focal contacts. When platelets were pre-incubated with BIM I, this translocation was largely abolished. A similar pattern of translocation occurred in response to thrombin (0.1 units/ml) for 4 min, although, in this case, inhibition of PKC with BIM I alone only partially blocked translocation of VASP. Inhibition of Rho kinase with Y27632 alone also had no significant effect upon translocation of VASP, but combined inhibition of PKC and Rho kinase largely ablated the translocation.

DISCUSSION

VASP is a critical protein that is involved in the remodelling of the actin cytoskeleton, and, in platelets, it clearly plays a role in regulating adhesive events that are involved in platelet aggregation [13,14]. It is well-established that VASP is phosphorylated by both PKA and PKG, and it has been suggested that phosphorylation at either Ser^{157} or Ser^{239} serves as an important regulatory mechanism [4,32]. In addition, however, Ser^{157} has been reported to be phosphorylated directly by PKC in cultured rat aortic smooth muscle cells [24]. In the present paper, we have reported both PKC-dependent and Rho kinase-dependent site-specific phosphorylation of VASP, on Ser^{157}, in response to stimulation of platelets by the physiological agonist thrombin. The observed phosphorylation may have important implications for VASP function in platelets.

Chitaley et al. [24] have shown that both serum and the phorbol ester PMA could induce a PKC-dependent phosphorylation of VASP in rat aortic smooth muscle cells, and so, in the first instance, we chose to determine whether PMA could induce a similar phosphorylation of VASP on Ser^{157} in human platelets, and whether this could be inhibited by the broad spectrum PKC inhibitor BIM I. Figure 1 shows that this was in fact the case and

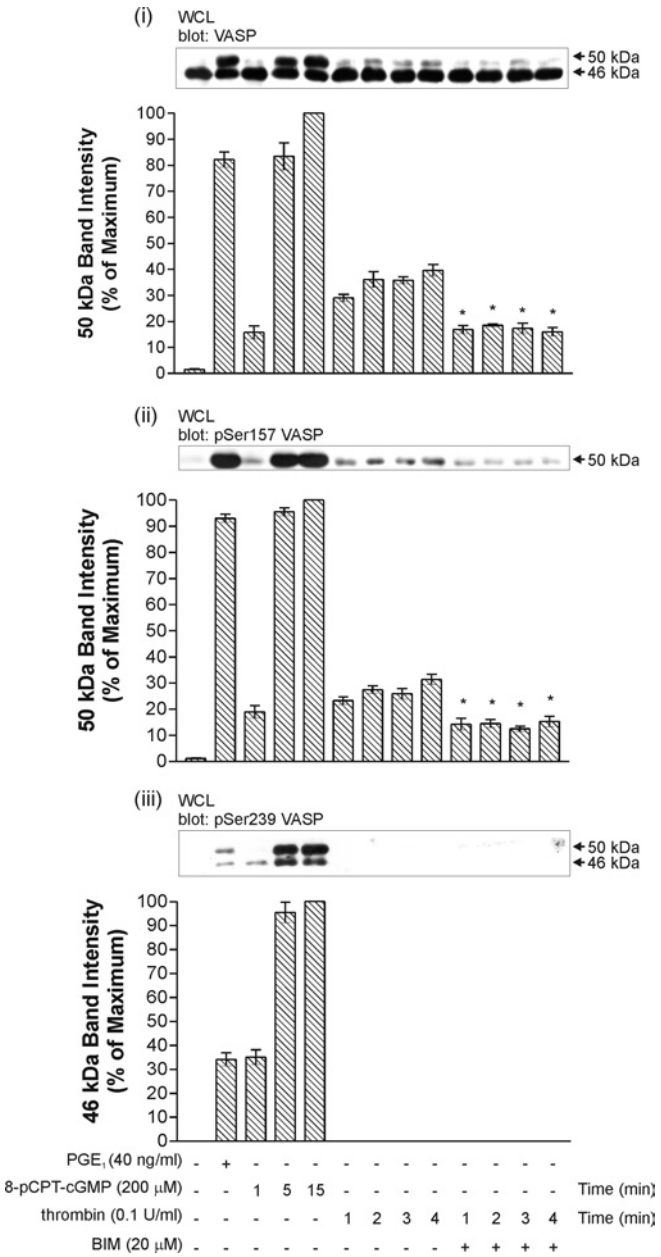

Figure 4 Thrombin induces phosphorylation of VASP on Ser[157], but not Ser[239], in a manner partially inhibited by BIM I

Platelets were pre-treated with BIM I (20 μM) or DMSO as vehicle control for 15 min before stimulation with thrombin (0.1 unit/ml) for various time periods (as indicated) in the presence of 1 mM EGTA. As positive controls for VASP phosphorylation, platelets were treated with either PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 1, 5 or 15 min. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were resolved by SDS/PAGE. VASP phosphorylation was detected by immunoblotting with polyclonal anti-VASP antibody (i), anti-pSer[157]-VASP antibody (ii) and anti-pSer[239]-VASP antibody (iii). Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 15 min. Results are means $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ compared with respective thrombin alone controls. WCL, whole-cell lysate.

that BIM I caused full inhibition of PMA-induced VASP phosphorylation on Ser[157], suggesting that PKC was the only kinase involved in mediating PMA-stimulated VASP phosphorylation. The possibility that BIM I could inhibit other kinases could not be ruled out, as it has been shown to inhibit GSK3 (glycogen synthase kinase-3), for example [33], although BIM I did not inhibit 8-pClPhs-cGMP-induced VASP phosphorylation (Figure 2B) and was therefore not inhibiting PKG. It was therefore possible that PKC was mediating phosphorylation of VASP directly, and it was important to attempt to identify the isoform of PKC that was involved in the response. In order to address this, we used a selective inhibitor of classical PKCs, Gö6976, which also inhibited PMA-induced VASP Ser[157] phosphorylation (results not shown). We propose that the kinase responsible is either PKCα or PKCβ, since PKCγ is very poorly expressed in platelets [34,35]. Our data are therefore in agreement with Chitaley et al. [24], who demonstrated the involvement of PKCα or PKCβ in phosphorylation of VASP in rat aortic smooth muscle cells. Despite the involvement of PKC in VASP phosphorylation, however, we were unable to show a direct interaction between these PKC isoforms and VASP in co-immunoprecipitation studies (J. K. T. Wentworth and A. W. Poole, unpublished work). It is therefore possible that the PKC-dependent phosphorylation is mediated by a PKC-dependent kinase, or equally possible that any interaction between PKC and VASP is transient and weak.

Following this, it was important to address whether a physiological agonist that couples to activation of PKC could also induce phosphorylation of VASP on Ser[157] in a PKC-dependent manner. We chose to assess the effect of the major platelet agonist, thrombin, upon VASP phosphorylation, and show that it too is able to induce phosphorylation of Ser[157], but not Ser[239], in platelets. In contrast with PMA, however, thrombin-induced phosphorylation of Ser[157] was only partially blocked by the PKC inhibitor BIM I. It was therefore important to try to determine the kinase responsible for PKC-independent phosphorylation of VASP by thrombin. We have provided evidence to show that the PKC-independent component does not involve PKG. First, the phosphorylation of VASP induced by thrombin occurred at Ser[157] and not Ser[239]. Activation of PKG, however, induces phosphorylation at both these sites, as we and others have shown (Figure 1; [20]), and therefore it would be expected that any PKC-mediated activation of PKG would lead to phosphorylation of both Ser[157] and Ser[239]. This also rules out the possibility that the major isoform of PKG expressed in platelets, PKG Iβ, is directly phosphorylated and activated by PKC, as has been shown for PKG Iα previously [36]. Secondly, it has been proposed that agonists such as thrombin may stimulate a rise in cGMP levels through activation of platelet calcium-dependent eNOS. This was ruled out by inhibition of sGC, using the inhibitor ODQ, which did not prevent VASP phosphorylation on Ser[157] (Figure 5B). We have not used selective inhibitors of PKG in the present study as there is mounting evidence to suggest that many of the commercially available pharmacological compounds do not significantly inhibit PKG [37–39], and their action is therefore non-selective, making it difficult to come to definitive conclusions using these compounds.

PI3K is known to become activated downstream of thrombin stimulation of platelets [40], and it was therefore possible that a PI3K-dependent kinase could be responsible for PKC-independent phosphorylation of VASP. This was ruled out, however, by the demonstration that the inhibitor wortmannin did not affect thrombin-induced VASP phosphorylation on Ser[157]. The nature of the PKC-independent kinase was therefore still not clear, but Rho kinase was a potential candidate because of its role in regulating the actin cytoskeleton. Overexpression of Rho kinase in HeLa cells has been shown to stimulate focal adhesion complexes and stress fibres [41]. In platelets, Rho kinase translocates to the Triton X-100-insoluble fraction after stimulation with thrombin, indicating a translocation to the cytoskeleton [42]. We, and others, have also shown a critical role for Rho kinase in mediating shape-change responses and actin rearrangement in platelets

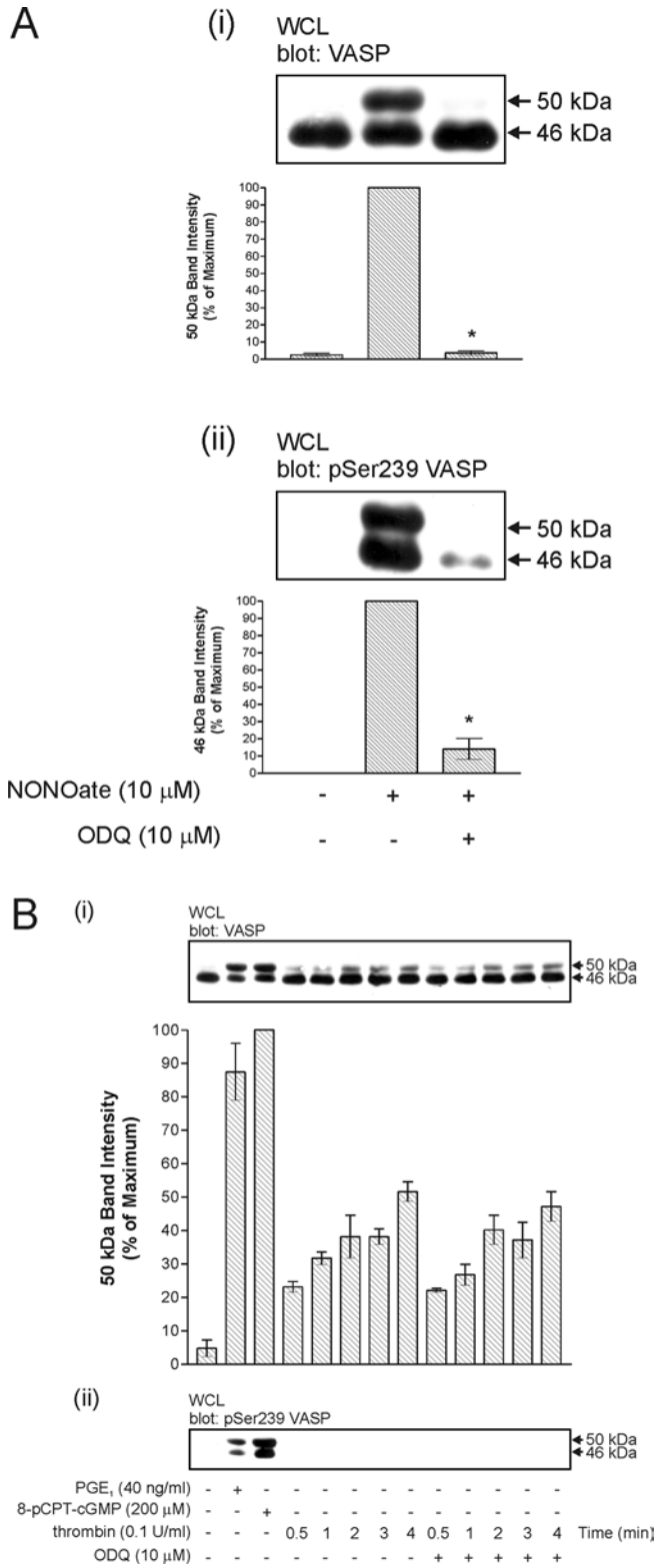

Figure 5 Thrombin-induced phosphorylation of VASP is not mediated by cGMP

(**A**) Platelets were pre-treated with the guanylate cyclase inhibitor ODQ (10 μM) for 20 min before the addition of NONOate (10 μM) for 2 min. Proteins were resolved by SDS/PAGE, and VASP phosphorylation was assessed by immunoblotting with anti-VASP-antibody (i) or anti-phosphoSer^{239} VASP-antibody (ii). *, $P < 0.05$ compared with NONOate alone control. (**B**) Platelets were pre-treated with the guanylate cyclase inhibitor ODQ (10 μM) for 20 min before stimulation with thrombin (0.1 unit/ml), for the times indicated. As positive controls for VASP phosphorylation, platelets were treated with either PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 5 min. VASP phosphorylation was assessed by immunoblotting with anti-VASP-antibody (i) or anti-phosphoSer^{239} VASP-antibody (ii). Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 5 min. Results are means $\pm$ S.E.M. ($n = 3$). WCL, whole-cell lysate.

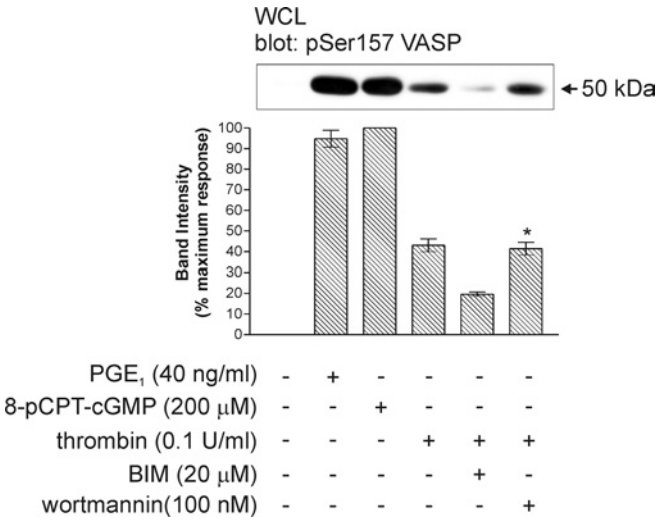

Figure 6 Thrombin-induced phosphorylation of VASP on Ser^{157} is not mediated by a PI3K-dependent pathway

Platelets were pre-treated with BIM I (20 μM), wortmannin (10 μM) or DMSO as vehicle control for 15 min before stimulation with thrombin (0.1 unit/ml) for 4 min. As a positive control for VASP phosphorylation, platelets were treated with PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 5 min. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were resolved by SDS/PAGE. Phosphorylation of VASP was detected by immunoblotting with anti-pSer^{157}-VASP antibody. Results shown in the upper panels are representative of three independent experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 5 min. Results are means $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ compared with thrombin alone control. WCL, whole-cell lysate.

[43–47], and, since both VASP and Rho kinase are involved in the rearrangement of the platelet actin cytoskeleton, we speculated that Rho kinase may also be responsible for phosphorylation of VASP. In the present study, we have shown that Rho kinase is able to phosphorylate VASP on Ser^{157} in response to stimulation of platelets with thrombin, and therefore represents an important non-PKC-dependent pathway. Pharmacological inhibition by H1152P and Y27632, two structurally distinct compounds, resulted in partial ablation of thrombin-induced VASP phosphorylation. The combination of either H1152P or Y27632 with BIM I almost completely abolished VASP phosphorylation on Ser^{157}, as immunodetected by anti-phosphoSer^{157} VASP-antibody. This confirmed that the two phosphorylation pathways, mediated by PKC and Rho kinase, were largely independent and therefore additive in nature, and showed that these two pathways account for the majority of phosphorylation of VASP Ser^{157} in response to thrombin.

It was then important to attempt to address the functional significance of phosphorylation of Ser^{157} on VASP. The precise functional role for the three distinct phosphorylation sites of VASP has not been elucidated. Phosphorylation of murine VASP on Ser^{153} and Ser^{235}, which are homologous with human VASP Ser^{157} and Ser^{239} respectively, was shown to inhibit F-actin interaction

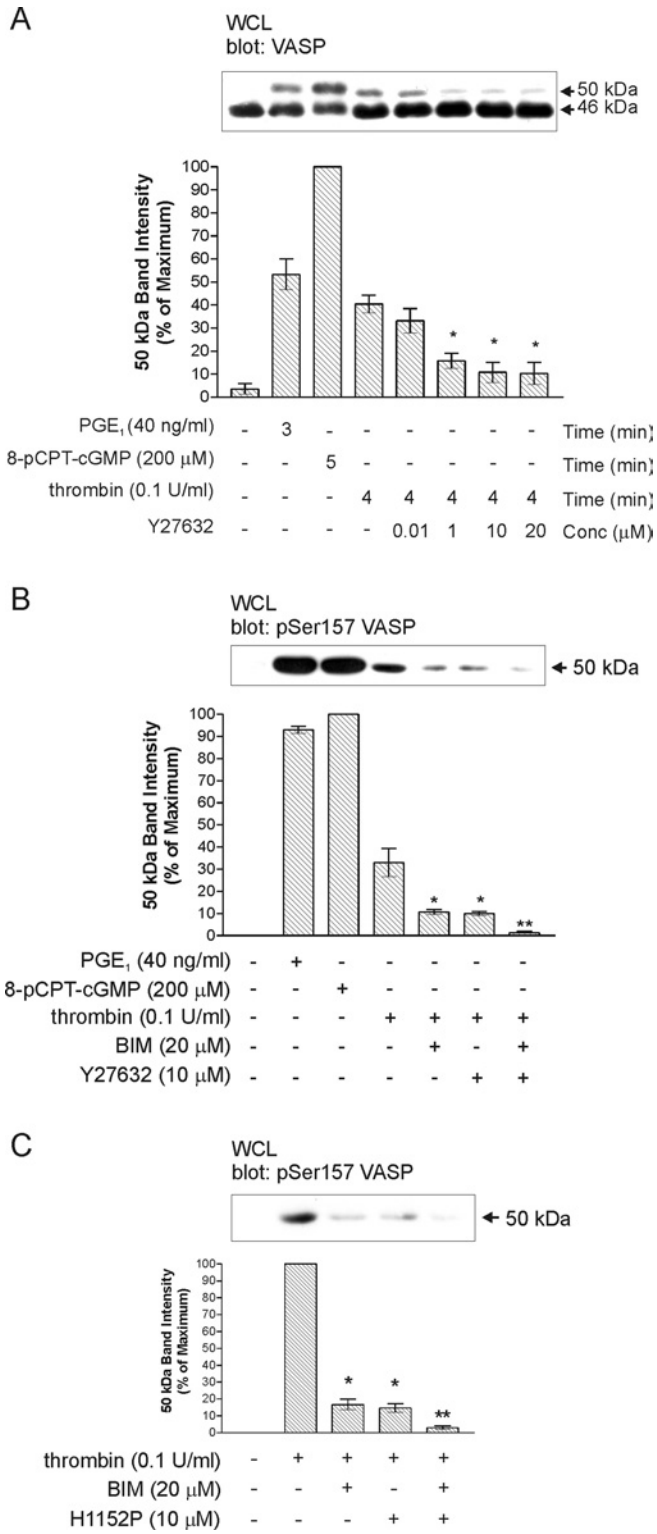

Figure 7 Thrombin-induced phosphorylation of VASP is partly mediated by Rho kinase

(**A**) Platelets were pre-treated with various concentrations of Y27632 for 25 min as indicated or DMSO as vehicle control before stimulation with thrombin (0.1 unit/ml) for 4 min. As a positive control for VASP phosphorylation, platelets were treated with PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (8-pCPT-cGMP; 200 μM) for 5 min. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were resolved by SDS/PAGE. Phosphorylation of VASP was detected by immunoblotting with anti-VASP antibody. (**B**, **C**) Platelets were pre-incubated with BIM I (20 μM) for 15 min, Y27632 (10 μM) for 25 min, H1152P (10 μM) for 25 min or DMSO as a vehicle control before stimulation with thrombin (0.1 units/ml) for 4 min. As a positive control for VASP phosphorylation in (**B**), platelets were treated with PGE_1 (40 ng/ml) for 3 min or 8-pCIPhs-cGMP (200 μM) for 5 min. Samples were lysed directly into 5-fold-concentrated sample solvent, and proteins were resolved by SDS/PAGE. Phosphorylation of VASP was detected by immunoblotting with anti-pSer^{157}-VASP antibody. Results shown in the upper panels are representative of three individual experiments. Bands from these experiments were quantified by densitometry, and results are represented in the lower panel histograms as percentages of maximal mean VASP phosphorylation in response to 8-pCIPhs-cGMP (200 μM) for 5 min for (**A**) and (**B**) or in response to thrombin (0.1 unit/ml) for 4 min for (**C**). Results are means $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ compared with thrombin alone control; **, $P < 0.05$ compared with thrombin plus BIM I or thrombin plus Y27632 (**B**), or compared with thrombin plus BIM I or thrombin plus H1152P (**C**). WCL, whole-cell lysate.

with VASP, actin nucleation and actin bundling *in vitro* [23]. Replacement of Ser^{153} and Ser^{235} with aspartate residues disrupted the ability of VASP to bundle, polymerize and nucleate actin, but did not inhibit activity to the same extent as that of phosphorylation by PKA. Phosphorylation on Ser^{153}, however, had no effect on the binding of VASP to its ligands zyxin, vinculin and profilin. EVL phosphorylation by PKA on Ser^{157}, its only phosphorylation site, was reported to reduce, but not completely inhibit, actin nucleation and polymerization [8]. Conversely, phosphorylation of VASP on Ser^{157} has been linked with an increase in affinity for actin, since Laurent et al. [48] have reported that the phosphorylated form of VASP displayed a 40% greater affinity for actin compared with unphosphorylated VASP. The role of Ser^{157} phosphorylation in regulating actin cytoskeleton rearrangement is therefore not clear.

Additionally, however, it has been shown that phosphorylation of VASP may also regulate cell proliferation. Chen et al. [49] have recently proposed that phosphorylation of VASP on Ser^{157} promotes the proliferation of vascular smooth muscle cells. This finding is consistent with the fact that VASP has been shown to be an important downstream component in RhoA-stimulated SRE (serum-response element)-dependent transcription, a process linked to cell proliferation [50]. In contrast, Chen et al. [49] also reported that phosphorylation of VASP on Ser^{239} by PKG inhibited smooth muscle cell proliferation and suggested that it is by this mechanism that $NO^{\bullet}$ inhibits the cellular proliferation of smooth muscle.

It was not possible in this series of experiments to definitively assign a role for phosphorylation of VASP at Ser^{157} by PKC or Rho kinase. We were, however, able to address whether these two kinases were able to regulate the localization of VASP upon platelet activation. Thrombin and PMA led to the translocation of VASP to more peripheral and punctate regions of the cell (Figure 8). It is interesting to speculate whether this punctate localization corresponds to the ends of actin filaments or focal adhesion points. BIM I was shown to abolish VASP translocation caused by PMA, indicating that PKC activity is important for translocation. However, in response to thrombin, the effect of BIM I was minimal. This is likely to be due to functional redundancy with Rho kinase; however, since, although blockade of Rho kinase alone also had minimal effect upon VASP translocation, the combined blockade of PKC and Rho kinase effectively abolished translocation. Although it is not possible from these experiments to elucidate whether phosphorylation of VASP at Ser^{157} regulates its translocation, there are likely to be other mechanisms also responsible, since it is clear that only a small proportion of VASP is phosphorylated in response to agonist, but clearly the majority of VASP translocates upon activation by thrombin. Additional signals, downstream of PKC and Rho kinase, are therefore likely to play a role in regulating localization of VASP in response to thrombin.

Finally, phosphorylation of VASP has been shown to regulate its ability to bind proteins to its proline-rich domain. Members

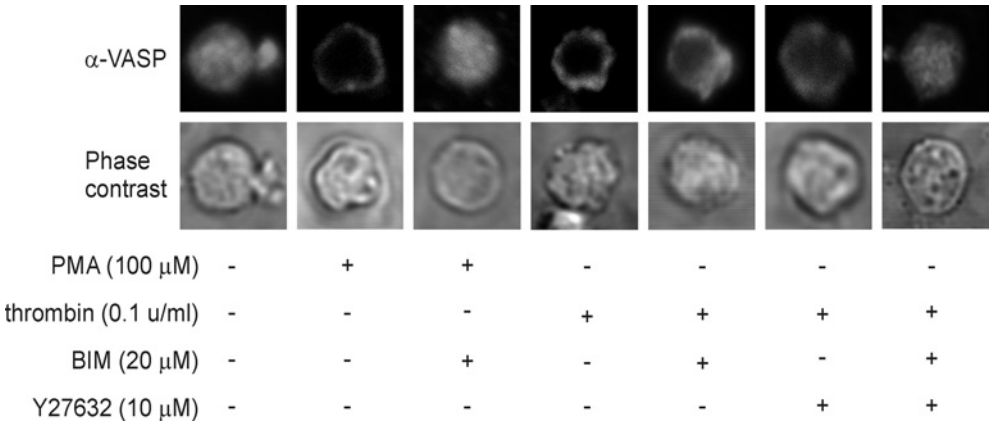

Figure 8 Thrombin-induced translocation of VASP is partially dependent upon PKC activity

Platelets were pre-treated with BIM I (20 μM), Y27632 (10 μM) or DMSO as vehicle control for 15 min before stimulation with either PMA (100 nM) for 5 min or thrombin (0.1 unit/ml) for 4 min in the presence of 1 mM EGTA. Platelets were processed for confocal immunofluorescence microscopy as detailed in the Experimental section, and fixed and stained with polyclonal anti-VASP antibody and an FITC-labelled anti-rabbit secondary antibody. Images shown are from a single experiment, but are representative of three repetitions.

of the Ena/VASP family have been shown to interact with SH3-domain-containing proteins, including members of the Src family of protein kinases, via the proline rich regions in VASP [8–11]. It has been suggested that phosphorylation of VASP at Ser^{157}, due to its proximity to the proline-rich domains, may disrupt interaction with SH3-domain-containing proteins. Phosphorylation of Ser^{157} on VASP, or Ser^{156} in EVL, serves to disrupt the association with SH3 domains, but does not affect the binding of profilin or EVH1-domain-interacting proteins [19,23]. In the present study, we used a co-immunoprecipitation approach to assess interaction with Fyn, Lyn or Src kinases, but could not reveal any interaction between non-phosphorylated VASP and these proteins in human platelets (results not shown), thus ruling out a role for Ser^{157} phosphorylation in regulating these interactions. We could not rule out the possibility, however, that other SH3-domain-containing proteins may interact with VASP, and that the interaction may be regulated by serine phosphorylation of VASP.

In conclusion, we have provided evidence that VASP is phosphorylated on Ser^{157} in platelets activated by thrombin. This phosphorylation is mediated by PKC-dependent and Rho kinase-dependent mechanisms, which may summate to achieve full phosphorylation of this residue. It will now be important to determine definitively the functional role of this phosphorylation in platelet activation.

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